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Oxidative stress in chronic obstructive pulmonary disease patients submitted to a rehabilitation program

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Summary
Pulmonary rehabilitation (PR) improves physical capacity and health quality in patients with chronic obstructive pulmonary disease (COPD). However, the effect of exercise on oxidative stress markers in COPD patients is only partially known. This study was designed to evaluate the oxidative stress response to long-term exercise in patients with COPD enrolled in a PR program. Fifteen COPD patients ($FEV_1 < 60\%$), age between 50 and 60 years, ex-smokers, were separated in two groups: exercise-trained ($n = 8$) and sedentary group ($n = 7$). Exercise consisted of an 8-week conditioning program using a cycle ergometer (three times a week, 1 h session). An endurance test (60% of maximal load in an incremental cycle test) was performed before and after PR. Blood samples were obtained at baseline and immediately after each endurance test. We measured the index of lipid peroxidation, thiobarbituric acid reactive species (TBARS), total radical-trapping antioxidant parameter (TRAP) and xanthine oxidase (XO) activity. TRAP was significantly different between the exercise-trained group and sedentary group of COPD patients. Baseline TBARS values were increased after the exercise training program but decreased after the endurance test. XO decrease after effort in the trained and untrained groups. The results suggest that patients with COPD are characterized by increased systemic and

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pulmonary oxidative stress markers both at rest as well as induced by cardiopulmonary exercise test and that PR program was associated with decreased systemic exercise-induced oxidative damage.

Introduction

Chronic obstructive pulmonary disease (COPD) has been considered by the organizations of health as one of the main morbidity and mortality causes in the world. It is characterized by airflow limitation that is not fully reversible.[1] The airflow limitation is in most cases both progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases.[2]

Improving the quality and duration of life of COPD patients is a distinct challenge. The specific therapeutic goals are to reduce symptoms, preserve lung function, optimize gas exchange, and limit and/or treat acute exacerbations rapidly.[3] Because pulmonary function declines with aging, everything must be done to prevent any additional functional loss caused by COPD. In general, the accepted strategy for stable patients consists of administering bronchodilators. Systemic corticosteroid for up to 2 weeks with or without antibiotics can be used to treat COPD exacerbation. Long-term inhaled corticosteroids can be considered for patients with multiple exacerbations. Additional therapy includes nutritional supplementation, immunization, supplemental oxygen, breathing exercises and pulmonary rehabilitation—PR.[4]

Chronic pulmonary disease can be viewed as a vicious cycle of disabling symptoms that lead to physical inactivity, deconditioning, and worsening symptoms of exercise limitation. However, PR program can be viewed as the strategy to break this cycle.[5]

Pulmonary rehabilitation is a multi-disciplinary program and is strongly suggested for the treatment of COPD, since PR increases functional capacity, decreases symptoms, reduces utilization of health care resources and improves quality of life.[4]

Although the benefits of physical exercise on pulmonary function in patients with COPD are limited, the physical training is the most important component of the pulmonary rehabilitation program. Both endurance and resistance muscular training improve muscle function and exercise tolerance in COPD.[6] Those evidences about the muscular function have a great importance in the treatment of COPD, because they are in agreement with the statement of the American Thoracic Society.[5] Evidence show that the exercise intolerance in COPD patients is related with the muscular dysfunction, that includes low muscle mass and strength, low muscle aerobic enzymes and capillarity, early onset of lactic acidosis, low VO_2 kinetics, among other effects.[7]

Oxidative stress has been suggested as a potential mechanism in the pathogenesis of COPD.[2] Evidence for increased oxidative stress in obstructive airway diseases is emerging and several studies have suggested that it can play an important role in their evolution and pathogenesis.[4] Reactive oxygen species (ROS) have long been implicated in adult respiratory distress syndrome (ARDS), in emphysema, and in COPD.[4] However, their importance in the pathogenesis of lung disease is not totally clarified since it is still difficult to obtain definitive proof that oxidative stress contributes to them.

Opposing to the benefits of the physical exercise on patient with COPD, evidences indicate that physical exercise, especially aerobic, generate ROS such as superoxide anion and hydrogen peroxide, capable of causing muscular damage and inflammation.[8] ROS are normally produced in the organism during metabolism, but may cause dangerous effects when produced excessively by reacting with cell components, including nucleic acids, proteins, and lipid. This process is known as oxidative damage.[9]

Physical exercise can induce an increase antioxidant defenses in the healthy people's organism, but effect of exercise on oxidative stress markers and the relationship among the production of free radicals and the capacity of antioxidant defense in COPD patients is only partially known. Thus, this study was designed to evaluate the oxidative stress response in patients with moderate and severe COPD enrolled in a RP program.

Material and methods

Subjects

Fifteen COPD patients ($FEV_1 < 60\%$), age between 50 and 60 years, ex-smokers, were separated in two groups: exercise-trained ($n = 8$) and sedentary group ($n = 7$). The patients were informed of the procedures involved and any possible risks and discomfort associated with the experiment before giving written consent. All procedures were approved by the local ethic committee.

Pulmonary function tests

All participants underwent flow volume tests, including measurements of FEV_1 and FVC, with the highest value from at least three properly performed measurements being used for analysis. The values obtained were expressed as a percentage of the reference value.[10]

Experiment protocol

Before training program, the patients performed a cardiopulmonary exercise test (CPET). An incremental exercise protocol in cycle ergometer (Ergomedic 828E, Monark) was used at a constant pedal speed of 60 rpm. Exercise power output was increased 5–10 W for every 2 min until voluntary cessation of exercise or until subjects were unable to maintain pedaling frequency. Expired air was monitored continuously during exercise using an on-line gas analysis system (*Total Metabolic Analysis System*, TEEM 100,

Aerosport, Ann Arbor, USA). Before and after the training protocol, the subjects also performed a continuous endurance test in cycle ergometer (Ergomedic 828E, Monark, Sweden) until voluntary cessation of exercise or until subjects were unable to maintain pedaling frequency. Endurance test workload was calculated based on CPET results to elicit an oxygen uptake of 60% maximum oxygen uptake. A sample of 10 ml heparinized blood was obtained from an antecubital vein at baseline and immediately after each endurance test. The blood was centrifuged (750 g, 10 min, 4 °C), the plasma was separated and stored at −80 °C until analysis.

In the training program (three times a week, 8 weeks) the subjects cycled at 60% peak maximum oxygen uptake for up to 1 h using cycle ergometer (Exercise cycle 827E, Monark, Sweden).

Total radical-trapping antioxidant parameter (TRAP)

Briefly, the reaction was initiated by adding luminol and ABAP (2,2′-azo-bis(2-amidinopropane)) in glycine buffer that resulted in steady luminescence emission. The addition of organ homogenate (150 μg of protein) decreases the luminescence proportional to the sample concentration of non-enzymatic antioxidants. Luminescence was measured in a scintillator counter.[11]

Thiobarbituric acid reactive species (TBARS)

As an indicator of lipid peroxidation, the formation of substances reactive to the heating of thiobarbituric acid (malondialdehyde—MDA) measured spectrophotometrically (532 nm) and expressed as malondialdehyde (MDA) equivalents.[12] Briefly, the samples were mixed with 1 ml of trichloroacetic acid 10% and 1 ml of thiobarbituric acid 0.67%, and then they were heated in a boiling water bath for 30 min.

Xanthine oxidase (XO) activity

XO activity was measured using oxidation of protein to isoxanthopterine.[13]

Protein determination

The amount of proteins in the assays of XO, TBARS and TRAP was assayed using the Lowry technique[14] and protein carbonyl by Bradford assay.[15]

Statistical analysis

Data are expressed as mean and the statistical method was assessed by a two-way analysis of variance (ANOVA) and Tukey post hoc comparison. The level of established significance used for all the statistical tests will be of $p<0.05$. The software used for analysis of the data was "Statistical Package for the Social Sciences (SPSS) version 12.0 for Windows".

Results

Clinical data

As shown in Table 1, anthropometric data were not significantly different between patients with COPD and control participants. The COPD group showed a moderate to severe airflow obstruction (FEV_1 of 35.8±10.1% predicted).

Total antioxidant capacity (TRAP)

Before the training program we did not determined differences between plasma TRAP before and after the effort test (Fig. 1). After training, patients presented lower plasma TRAP levels when compared to untrained group (Fig. 2). This indicates a consumption of non-enzymatic antioxidant defenses after program training.

Lipid peroxidation

The oxidative damage to membrane lipids were evaluated by the formation of MDA, a sub-product for lipoperoxidation. Before effort in the trained group, TBARS values were increased when compared to both baseline and untrained pre-effort (Fig. 3). These results reinforced TRAP values, indicating that training induces oxidative damage in COPD patients.

Table 1 Baseline characteristics of patients with chronic obstructive pulmonary disease before the pulmonary rehabilitation program.

	All patients with COPD (mean±SD)	All patients with COPD—trained (mean±SD)	All patients with COPD—untrained (mean±SD)	*p*
Age (%)	66.1±5.7	66.4±3.0	65.9±7.6	0.86
BMI (kg/m^2)	26.8±3.2	27.6±3.4	26.1±3.1	0.39
FVC (%) predicted	60.2±12.3	56.3±12.9	63.6±11.4	0.26
FEV_1 (%) predicted	35.8±10.1	34.0±9.9	37.4±10.6	0.54
FEV_1/FVC	46.9±7.9	46.4±4.7	47.2±10.2	0.85

Definition of abbreviations: BMI: body mass index; COPD: chronic obstructive pulmonary disease; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity. Data are presented as mean±SD ($^*p<0.05$).

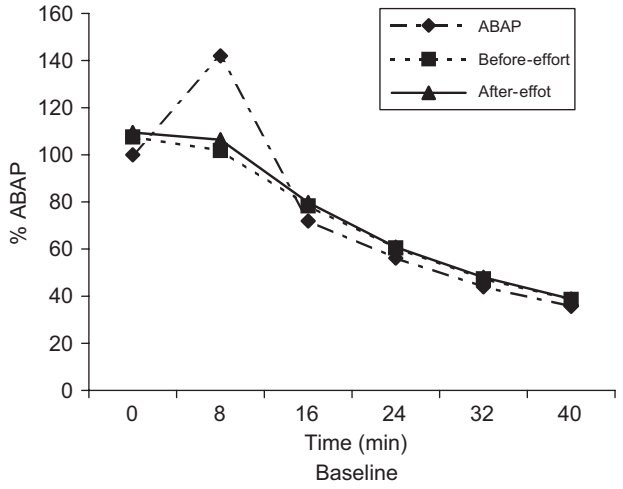

Figure 1 Total antioxidant capacity (TRAP) of patients with chronic obstructive pulmonary before and after the effort test. The methodological proceedings are described in the Material and Methods section. The values are presented as MEAN ± SEM. Significant different between groups ($p < 0.05$).

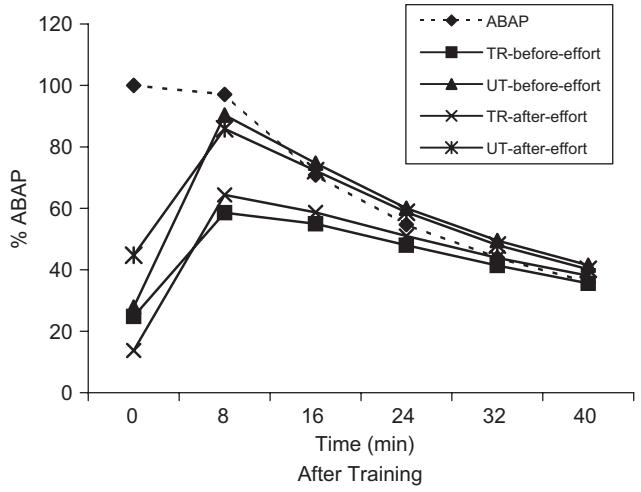

Figure 2 Total antioxidant capacity (TRAP) of patients with chronic obstructive pulmonary after pulmonary rehabilitation program and before and after the effort test. The methodological proceedings are described in the Material and Methods section. The values are presented as MEAN ± SEM. Significant different between groups ($p < 0.05$).

XO

We observed lower XO activity values in all groups after endurance test and lower XO activity values before endurance test in trained group compared to the untrained (Fig. 4).

Discussion

The objective of this study was to evaluate the oxidative stress response to long-term exercise in patients with moderate and severe COPD enrolled in a pulmonary rehabilitation program. There is accumulating evidence that oxidative stress may play an important role in COPD.[6,16,17] In the same way, physical exercise, especially aerobic, generated reactive oxygen species capable of causing muscular damage and inflammation.[8] However, an elaborated oxidative defense system results from a regular physical exercise program.[9]

In COPD patients, the presence of oxidative stress has been assessed by measuring markers of the effects of radicals on lung biomolecules and/or by measuring the stress responses to the increased oxidant burden.[2]

Although some authors have described the metabolic pathway of the production of ROS during exercise, the mechanism of the oxidative stress and the influence of the physical conditioning on the liberation of ROS are little known, mainly in people with COPD.

In order to access the non-enzymatic antioxidant status, the TRAP assay was performed. Initially, we demonstrated a consumption of non-enzymatic antioxidant defenses in people with COPD after training program (Fig. 1). TRAP has provided a sensitive tool to quantify combined non-enzymatic antioxidant capacity of plasma or tissues. Among those nonenzymatic antioxidants, it is well known that glutathione plays an important role in the defense mechanism of the lung.[18]

The glutathione is concentrated in epithelial lining fluid (compared with plasma) and appears to have an important protective role, together with its redox enzymes in the airspaces and intracellularly in epithelial cells.[19]

Low non-enzymatic antioxidant capacity after the training program before and after the endurance test in trained and untrained groups (Fig. 2) can be associated with the oxidation of glutathione induced by physical exercise. Viña and colleague showed that exercise-induced glutathione oxidation occurs in patients with COPD as well as in healthy subjects.[20] It is probable that this decrease of the antioxidant capacity is due to reduction in the consumption of oxygen during exercise that frequently happens in patients with COPD, decreasing the formation of ROS. The oxidative stress in patients with COPD during or immediately after moderate or intense physical effort includes disturbances in the mitochondrial breathing chain as well as a contribution of other sources of ROS generated during the exercise.[21]

We also determined lipid peroxidation as measurement of cellular damage in COPD patients. Lipid peroxidation, resulting from the reaction of free radicals with polyunsaturated fatty acid side chains in membrane lipoproteins, is a further reaction that can result in cell damage, and is a self-perpetuating process that continues as chain reactions. The results show that before the endurance test in the trained group, the MDA level increased compared with both baseline values and untrained group (Fig. 3). This result is in agreement with previous studies.[21] Agacdikem and colleagues investigated the effects of exercise on MDA levels in COPD patients and found that exercise acute caused significant increased in MDA level.[22] In the present study, we did not observe significant differences in MDA levels after the endurance tests. Our findings suggest that high plasma TBARS may be associated with lung function not only in smokers without COPD, but in patients with moderate COPD as well. These observations indicate that lipid

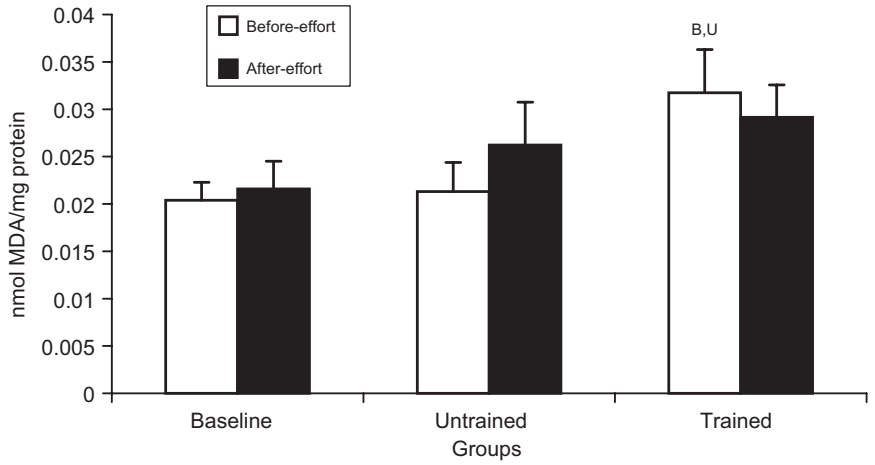

Figure 3 Lipoperoxidation level of patients with chronic obstructive pulmonary before pulmonary rehabilitation program (baseline) and before and after the effort test. Values are presented as MEAN ± SEM and the results were expressed in nmol MDA per milligram of protein. Significant different between baseline (^{B}) and untrained (^{U}) groups was $p < 0.05$.

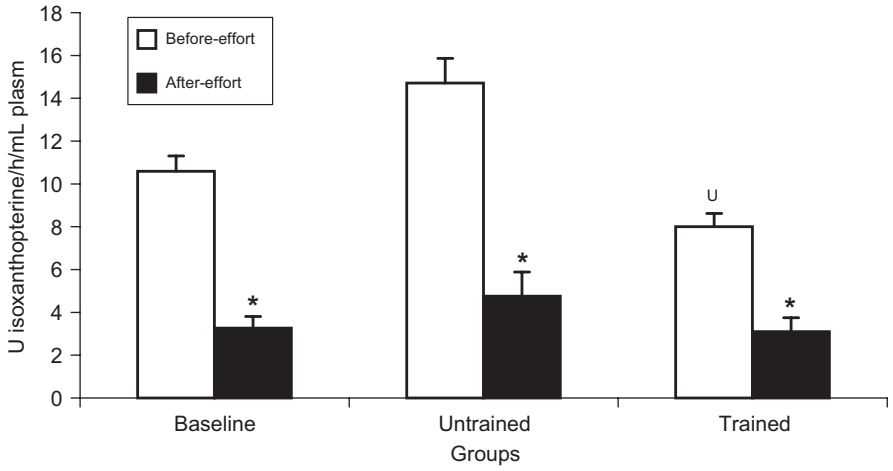

Figure 4 XO activity in patients with chronic obstructive pulmonary before pulmonary rehabilitation program (baseline) and before and after the effort test. Values are presented as MEAN ± SEM and the results were expressed in unit of isoxanthopterina per hour per ml plasma. Significant different in the group (*) and in relation to group untrained (^{U}) was $p < 0.05$.

peroxidation is markedly increased in patients with moderate COPD, in agreement with previous findings showing elevated levels of other markers of lipid peroxidation such as urinary and plasma concentrations of 8-8 isoprostane and exhaled ethane in patients with COPD.[23,24]

We evaluated XO activity, an enzyme that catalyzes the degradation of the hypoxanthine to xanthine and uric acid using the molecular oxygen as receiver of electrons, during the ischemic muscular work, taking to the increase in the production of O_2.[25] Generation of superoxide by XO plays an important role in ischemia/reperfusion injury. Expression of this enzyme has been seen in the peripheral and respiratory muscles of rodents and in the peripheral skeletal muscle of humans. Heunks and colleagues suggested that strenuous exercise in patients with COPD results in exercise-induced oxidative stress that is accompanied by tissue damage and that the XO contributes to free radicals generation during exercise.[26] Patients with moderate and severe COPD present a reduced threshold of muscular fatigue, due to decreased capacity of breathing muscles in maintaining a level of enough ventilation to assure normal alveolar ventilation, which can commit the appropriate elimination of CO_2.[21] One effect of acute exercise is also to increase PO_2 in the circulation, taking patient with COPD to a smaller adaptation to the effort. This metabolic demand of larger oxygen volume in these patients is associated to the increase in the concentration of XO with consequent production of anion superoxide.[22] We believed that those results would be reproduced in our study. However, our results (Fig. 4) surprisingly show that acute exercise (endurance test) reduced the values of XO in all the groups and that the trained group had their basal values significantly reduced. These differences could be related to different exercise protocols.

In conclusion, the present study suggested that patients with COPD are characterized by increased systemic and

pulmonary oxidative stress markers both at rest as well as induced by cardiopulmonary exercise test. Moreover, this study showed for the first time that supervised pulmonary rehabilitation was associated with decreased systemic exercise-induced oxidative damage.

References

1. Pauwels RA, Buist AS, Calverley PM, Jenkins CR, Hurd SS. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) workshop summary. *Am J Respir Crit Care Med* 2001;**163**:1256–76.
2. Gerritsen WBM, Asin J, Zanen P, Bosch JMM, Haas FJLM. Markers of inflammation and oxidative stress in exacerbated chronic obstructive pulmonary disease patients. *Respir Med* 2005;**99**: 84–90.
3. Spencer H. *Pathology of the lung*, 5th ed. Philadelphia: McGraw-Hill; 1996.
4. Celli B, Goldstein R, Jardim J, Knobil K. Future perspectives in COPD. *Respir Med* 2005;**99**:S1–S48.
5. Crapo RO, Casaburi R, Coates AL, et al. Guidelines for methacholine and exercise challenge testing-1999. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors. *Am J Respir Crit Care Med* 2000;**161**: 309–29.
6. Mercken EM, Hageman GJ, Schols AMWJ, Akkermans MA, Bast A, Wouters EFM. Rehabilitation decreases exercise-induced oxidative stress in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2005;**172**:994–1001.
7. Stevenson CS, Koch LG, Britton SL. Aerobic capacity, oxidant stress, and chronic obstructive pulmonary disease: a new take on an old hypothesis. *Pharmacol Therap* 2006;**110**:71–82.
8. Liu J, Yeo HC, Overvik-Douki E, Hagen T, Doniger SJ, Chyu DW, et al. Chronically and acutely exercised rats: biomarkers of oxidative stress and endogenous antioxidants. *J Appl Physiol* 2000;**89**:21–8.
9. Polidori MC, Mecocci P, Cherubini A, Senin U. Physical activity and oxidative stress during aging. *Inter J Sports Med* 2000;**21**: 154–7.
10. Quanjer PH, Tammeling GJ, Cotes JE, Pedersen OF, Peslin R, Yernault JC. Lung volumes and forced ventilatory flows. Report working party standardization of lung function tests, European Community for steel and coal. Official Statement of the European Respiratory Society. *Eur Respir J Suppl* 1993;**16**:5–40.
11. Tsal K, Hsu T-G, Kong CW, Lin KC, Lu FG. Is the endogenous peroxyl-radical scavenging capacity of plasma protective in systemic inflammatory disorders in humans? *Free Radic Biol Med* 2000;**28**:926–33.
12. Draper HH, Hadley M. Malondialdehyde determination as index of lipid peroxidation. *Meth Enzymol* 1990;**186**:421–31.
13. Beckman JS, Parks DA, Marshall PA, Freeman BA. A sensitive fluorometric assay for measuring xanthine dehydrogenase and oxidase in tissues. *Free Rad Biol Med* 1989;**6**:607–15.
14. Lowry OH, Rosebough NG, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; **193**:265–75.
15. Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* 1976;**72**:248–54.
16. Repine IE, Bast A, Lankhorst I. Oxidative stress in chronic obstructive pulmonary disease. The oxidative stress study group. *Am J Respir Crit Care Med* 1997;**156**:341–57.
17. MacNee W, Rahman I. Is oxidative stress central the pathogenisis of chronic obstructive pulmonary disease? *TRENDS Mol Med* 2001;**7**:55–62.
18. Zhang Z, Shen H-M, Zhang Q-F, Ong C-N. Critical role of GSH in silica-induced oxidative stress, cytotoxicity, and genotoxicity in macrophages. *Am J Physiol* 1999;**277**:L743–8.
19. Rahman I, MacNee W. Oxidative stress and regulation of glutathione in lung inflammation. *Eur Respir J* 2000;**16**:534–54.
20. Vinã J, Servera E, Asensi M, et al. Exercise causes blood glutathione oxidation in chronic obstructive pulmonary disease: prevention by O_2 therapy. *J Appl Physiol* 1996;**81**:2199–202.
21. Heunks LMA, Dekhuijzen PNR. Respiratory muscle function and free radicals: from cell to COPD. *Thorax* 2000;**55**:704–16.
22. Agacdiken A, Basyigit I, Ozden M, et al. The effects of antioxidants on exercise-induced lipid peroxidation in patients with COPD. *Respirology* 2004;**9**:38–42.
23. Pratico D, Basili S, Vieri M, Cordova C, Violi F, Fitzgerald GA. Chronic obstructive pulmonary disease is associated with an increase in urinary levels of isoprostane F2alpha-III, an index of oxidant stress. *Am J Respir Crit Care Med* 1998;**158**: 1709–14.
24. Paredi P, Kharitonov SA, Leak D, Ward S, Cramer D, Barnes PJ. Exhaled ethane, a marker of lipid peroxidation, is elevated in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2000;**162**:369–73.
25. König D, Berg A. Exercise and oxidative stress: is there a need for additional antioxidants. *Österreichisches J Für Sportmedizin* 2002;**3**:6–15.
26. Heunks LM, Vina J, van Herwaarden CL, Folgering HT, Gimeno A, Dekhuijzen PN. Xanthine oxidase is involved in exercise-induced oxidative stress in chronic obstructive pulmonary disease. *Am Physiol Soc* 1999;**277**:1697–704.